August 5, 1998

VETERINARY SERVICES MEMORANDUM NO. 800.90

Subject: Guidelines for Veterinary Biological Relative Potency Assays and Reference Preparations Based on ELISA Antigen Quantification

To: Veterinary Biologics Licensees, Permittees, and Applicants
Director, Center for Veterinary Biologics-Inspection and Compliance
Director, Center for Veterinary Biologics-Laboratory
Director, Center for Veterinary Biologics-Licensing and Policy Development
Director, National Veterinary Services Laboratories

I. PURPOSE

This document provides guidelines for conducting *in vitro* enzyme immunoassays to determine the relative antigen content (potency) of serials of inactivated veterinary biologicals as provided in Title 9, Code of Federal Regulations, Part 113, Section 113.8 (9 CFR 113.8).

II. INTRODUCTION

These guidelines provide parameters and procedures for conducting an enzyme-linked immunosorbent assay (EIA or ELISA) for the quantitation of antigen in a veterinary biological product. The guidelines also establish uniformity for such *in vitro* testing. In the ELISA or EIA system described, the concentration of a test serial is determined by comparing the absorbance values (optical density or OD) of that serial to the OD values of a reference preparation (Master or Working Reference). The relative potency of the test serial is determined from the relationship of the OD's. These guidelines address the following: definitions, immunogen test specificity, *in vitro* test design, reference qualification, reference requalification, Qualifying Serials, reference storage, reference dating, testing adjuvanted product, and statistical methods.

III. DEFINITIONS

A. Applicable 9 CFR definitions include:

1. Master Reference, 9 CFR 101.5(o). A Master Reference is a reference whose potency is correlated, directly or indirectly, to host animal immunogenicity. The Master Reference may be used as the working reference in *in vitro* tests for relative potency. The Master Reference may also be used to establish the relative potency of a serial of product used in requalification studies and to establish the relative potency of working references. The Master Reference as described in a filed Outline of Production may be:

a. A product reference that is a completed serial of vaccine or bacterin prepared in accordance with a filed Outline of Production. Product references may be:

i. Monovalent references composed of a single fraction or agent, or

ii. Polyvalent references composed of two or more fractions or agents.

b. A purified preparation of a protective immunogen or antigen, or

c. A nonadjuvanted harvested culture of microorganisms.

2. Working Reference, 9 CFR 101.5(p). A Working Reference is the reference preparation that is used in the *in vitro* test for the release of serials of product. Working References may be:

a. Master References, or

b. Serials of product that have been prepared and qualified, in a manner acceptable to the Animal and Plant Health Inspection Service (APHIS), for use as reference preparations.

3. Qualifying Serial, 9 CFR 101.5(q). A Qualifying Serial is a serial of biological product used to test for immunogenicity when the Master or Working Reference is a purified antigen or nonadjuvanted harvest material. Qualifying Serials shall be produced in accordance with the filed Outline of Production, tested for immunogenicity in accordance with methods deemed appropriate by APHIS, and have a geometric mean relative potency, when compared to the Master Reference, of not greater than 1.0 as established by independent parallel line assays with five or more replicates or by other valid assay methods for determining relative antigen content which demonstrate linearity, specificity, and reproducibility at least equivalent to the parallel line assay and are acceptable to APHIS. Qualifying Serials used to requalify or extend the dating period of a Master Reference shall be determined to be immunogenic in accordance with methods deemed appropriate by APHIS as provided in 9 CFR 113.8(a) and, in addition, shall be within their permitted dating period and have been prepared in accordance with the production method described in the currently filed Outline of Production.

4. Immunogenicity, 9 CFR 101.5(r). The ability of a biological product to elicit an immune response in animals as determined by test methods or procedures acceptable to APHIS.

B. <u>Definitions of other terms used in this Memorandum are as follows:</u>

1. Relative potency. Potency of a product as determined by comparison with an approved reference. For *in vitro* antigen potency assays, the unknown is compared with a working reference.

2. Protective immunogen. A known, characterized antigen that elicits an immune response in animals demonstrated to be directly correlated to protection or that supports the label claim in the host animal.

3. Test serial. A serial of product being evaluated for potency.

4. Critical reagents. The reagents in a test system that are key to detection and specificity of the assay. Usually consist of capture antibody, detection antibody, and/or competitive antigen. Critical reagents are usually lot-controlled.

5. Competitive test or assay. An assay in which binding of an antigen-enzyme conjugate to a solid-surface-bound antibody is inhibited (via competition for binding sites) in the presence of the unknown antigen. The greater the amount of antigen in the unknown, the less the color development.

6. Noncompetitive test or assay. An assay in which the binding of the antigen in the test serial to a solid-surface-bound antibody is in proportion to the amount of antigen present. The greater the amount of antigen in the test serial, the more the color development.

7. Direct test or assay. An assay in which the antigen in the test serial is directly adhered to a solid surface. The greater the amount of antigen bound to the solid surface, the more the color development.

8. Dose response. Ability of an assay or an animal model to respond to changes in concentration of the fraction being tested.

9. Reference qualification. Establishing the correlation of efficacy to potency for a new Master Reference. For reference qualification the criteria are demonstration of protection or a protective response which directly supports label claims.

10. Reference requalification. Reestablishing the potency/efficacy correlation of the Master Reference. For reference requalification the criterion is demonstration of the immunologic stability of the reference.

11. Reference standard. A reference that has been prepared and validated under the auspices of a National Control Authority or recognized Standards Organization and is used to standarize the level of reactivity of other references.

12. Reference degradation. Decrease in specificity and strength of an immune response as compared to the initial assay. Degradation may be caused by

temperature, light, time, and other adverse storage conditions.

13. Protective antigen(s). The antigen(s) that elicit(s) a protective immune response in host animals or support(s) label claims in a manner acceptable to APHIS.

IV. DETERMINING THE SPECIFICITY OF THE TEST SYSTEM

A. Prior to the establishment of a testing method in the filed Outline of Production or Special Outline, the following should be supplied to and approved by APHIS:

1. Evidence that the *in vitro* relative potency test measures a protective immunogen as demonstrated by one of the following methods:

a. Host animal studies using a purified subunit (e.g., glycoprotein or bacterial outer membrane antigen extract) that elicits protection against the specific animal disease,

b. Passive protection by the monospecific antibody component(s) of the *in vitro* test system,

c. Data published in peer reviewed scientific journals generally recognized by the scientific community and acceptable to APHIS,

d. Demonstration of *in vitro* neutralization of viable organisms by the detecting reagent, or

e. Other methods acceptable to APHIS.

2. Demonstration of the specificity of the reaction between a protective immunogen and the detection antibody by polyacrylamide gel electrophoresis/immunoblotting or other similarly specific techniques.

B. In general, the preferred ELISA or EIA potency test system uses as the detecting agent a monoclonal antibody specific for a protective immunogen. In preferred order, examples of acceptable techniques are:

1. Polyclonal capture/monoclonal detection assays (sandwich: polyclonal antibody adhered to the plate/antigen capture/antibody detection),

2. Monoclonal capture/monoclonal detection assay,

3. Competitive assays (antigen competing with the antigen being measured),

4. Monoclonal capture/polyclonal detection assays. The specificity of the

assay relative to the detection antibody should be evaluated prior to using this assay design,

5. Polyclonal capture/polyclonal detection assays. The specificity of the assay relative to the detection antibody should be evaluated prior to using this assay design, or

6. Direct assays (antigen adhered to the plate/antibody detection).

C. Antiserum (polyclonal or monoclonal antibody) that reacts with a nonprotective antigen, or with both protective and nonprotective antigens, should not be used as a critical detection reagent.

D. The specificity of the assay for the test antigen must be demonstrated when the test is validated and each time a critical reagent is changed; i.e., the presence of other fractions, serum, cellular proteins, etc., should not result in detectable reactions. During initial establishment of a test and each time a critical reagent is changed, it should be demonstrated that there is no crossreactivity with antigenic components other than the immunogen (i.e., other fractions, serum, cellular proteins).

E. Critical reagent(s) should be lot-controlled, should have stated storage conditions, and the method of use should be described in a filed Outline of Production or Special Outline.

V. TEST DESIGN

A. The entire assay for a single test (reference, test serial, blank, and controls) should be conducted on one (1) plate.

B. The reference, test serial, and controls should each have a minimum of two (2) wells per dilution. The use of three (3) or more wells per dilution may allow for the removal of outliers.

C. A minimum of three (3) dilutions are necessary to establish a statistically significant linear regression. Increasing the number of dilutions increases the likelihood of obtaining regression line(s) with satisfactory correlation coefficients and similar slopes.

D. Dilution schemes other than linear are acceptable. The dilutions used for the test serial need not be the same as the dilutions used for the reference.

E. More than one serial may be assayed on a single plate if space allows.

F. A blank consisting of at least one well (two [2] or more wells are recommended) should be included on each plate. For noncompetitive tests, blank wells should receive the same buffers, substrate, chromogen, etc., as the other wells, and the

mean OD value of the blank(s) should be subtracted from all other OD values before any data are analyzed.

G. The assay should measure reactions at both ends of the dose-response curve (i.e., antigen saturation and antigen extinction). The test should be designed for maximum dose response (steepest slope) so that the reference and test serial produce OD values within the working and/or midrange of the sensitivity of the test. Reevaluation of the assay is indicated if OD's and/or the slope of the line consistently fall outside established test parameters.

H. Positive and negative controls to monitor test performance and validate the test are recommended.

1. A positive control of known antigen concentration and established test parameters is used to verify consistency from test to test and may be a routine serial, purified antigen, or harvest culture. An acceptable range or OD for the positive control should be established.

2. A negative control is used to ensure that the observed reaction is due to the presence of the immunogen. A negative control typically contains all components of the test serial except for the immunogen. For noncompetitive tests the negative control can be used as the blank for the test. A maximum noncorrected OD for the negative control should be established.

3. If controls are used to determine the validity of a test (i.e., to declare a "no test"), their use must be described in the Outline of Production or Special Outline.

4. Controls must have unique lot control numbers, have stated validity criteria, and be available to the Center for Veterinary Biologics-Laboratory (CVB-L) upon request.

VI. REQUIREMENTS FOR A REFERENCE

A. The antigen content of a Master Reference should directly or indirectly correlate to protection of the host animal or support label claims.

1. A direct correlation is established when the Master Reference is used in the host animal protection study.

2. An indirect correlation is established when a Qualifying Serial is tested in host animals or when a Master Reference or Qualifying Serial is administered to animals other than host animals.

B. The reference used in the *in vitro* test for serial release is by definition a Working Reference but can, in addition, be the Master Reference.

C. It is recommended that the Master Reference and/or Working Reference be a product reference.

1. A product reference will have all the components in the same relative proportions as are found in the serial of product being tested.

2. A product reference can be either a monovalent reference or a polyvalent reference.

a. If a monovalent reference is used to evaluate a polyvalent product, it is unacceptable to compensate mathematically for *in vitro* interference occurring between fractions in the test serial.

b. If using a polyvalent reference to test product with fewer fractions than the reference, it is necessary to demonstrate that *in vitro* interference between fractions is not occurring. If *in vitro* interference occurs, then a monovalent reference or a reference with similar antigen content is required.

D. Purified references are acceptable, provided that:

1. The test system is not influenced by the other components present in the normal product,

2. Linearity and parallelism between the purified Master or Working References and the product being tested are maintained,

3. A Qualifying Serial is used to establish the purified Master Reference, and

4. There is no selective addition of components or compounds to a purified reference to compensate for nonlinearity or lack of parallelism.

E. Master References should be established to have a relative potency (RP) of one (1.0). This may be accomplished as follows:

1. For nonfrozen product references the RP of 1.0 is based on the concentration of antigen in the immunogenicity serial which demonstrated a protective response or supports label claims in a host animal immunogenicity trial.

2. For purified concentrated Master or Working References (frozen or non-frozen), the dilution of the reference equivalent to an RP of 1.0 is established at the time of reference qualification using a Qualifying Serial which demonstrates a protective response or supports label claims in a host animal immunogenicity trial.

a. Dilution of the purified Master or purified Working Reference prior to use in the test system should not exceed 1:100 and should be done with an inert-ingredient diluent (phosphate buffered saline, Dulbecco's phosphate buffered saline, physiological saline, water, etc.).

b. Alternative dilution procedures should be justified by data approved by APHIS and described in a filed Outline of Production or Special Outline.

3. Frozen product Master References should be established to have an RP of 1.0 and validated during qualification by the use of a qualifying product serial demonstrating a protective response or supporting label claims in a host animal immunogenicity trial.

a. Dilution of a product Master Reference with an inert ingredient diluent (phosphate buffered saline, Dulbecco's phosphate buffered saline, physiological saline, water, etc.) to compensate for a difference in dose is allowed.

b. Alternative procedures for frozen product references (mathematical correction, dilution of a frozen reference, or use of noninert-ingredient diluent, etc.) should be justified by data, approved by APHIS, and detailed in a filed Outline of Production or Special Outline.

F. It is recommended that a new vial of reference be used for each test. Vials of frozen references should not be refrozen or used for more than one (1) day unless the additional time or freeze-thaw cycles are supported by data submitted and approved by APHIS and the criteria specified in the Outline of Production or Special Outline.

G. When comparing a test serial to the Master Reference by a relative potency method, a satisfactory test should have a minimum relative potency greater than or equal to one (m1.0).

1. A relative potency of 1.0 is based on the antigen concentration of the Master Reference or Qualifying Serial of vaccine administered to host animals during an efficacy trial and the demonstration of protection meeting appropriate 9 CFR criteria or label claims.

2. Tests with a relative potency value greater than or equal to one (m1.0 or m 0.95) are considered satisfactory for a test serial at product release, during dating, or at the end of dating.

3. Antigen overage at product release may be required to ensure maintenance of the antigen concentration at 1.0 relative potency throughout product dating and should be taken into consideration during each serial

formulation.

H. The Master and/or Working Reference should be uniquely identified by lot number and expiration date in the filed Outline of Production or Special Outline.

1. The Outline of Production or Special Outline should specify the reference (including lot number) used for testing.

2. The Master Reference should be available in sufficient quantities to allow the manufacturer and the CVB-L to conduct testing throughout the dating of the reference and for an additional period if requalification is anticipated.

VII. QUALIFICATION OF A REFERENCE

A. Qualification of a Master Reference for use in *in vitro* relative potency assays is either the initial establishment of a Master Reference or the establishment of a new Master Reference to replace an existing reference. The dating of the Master Reference is for a defined time period as supported by data and approved by APHIS.

B. Master References that are unfrozen product references stored similarly to product can be directly qualified in animals.

C. Master References that cannot be directly qualified in animals, but require a Qualifying Serial for qualification, include:

1. Master References that are frozen product references,

2. Master References that are stabilized and frozen, and

3. Purified Master References.

D. The immunogenicity of new and replacement Master References should be established as prescribed in the applicable 9 CFR Standard Requirement or, for agents without codified requirements, in accordance with protocols acceptable to APHIS. Alternative immunogenicity proposals for qualifying Master References may be considered by APHIS, but should:

1. Include a statistically significant number of animals as would be required for initial licensure of a product,

2. Include an assessment of immunogenicity and/or efficacy of a product in the host species,

3. Be directly correlated to protection or support label claims, and

4. Be submitted to the firm’s assigned reviewer in the Center for

Veterinary Biologics-Licensing and Policy Development (CVB-LPD) soon enough to allow for comment and approval prior to test initiation.

E. Agent-specific alternative test methods for qualifying or requalifying Master References will be developed and provided as they become available.

VIII. REQUALIFICATION OF A REFERENCE

A. Requalification of a Master Reference requires demonstration of the immunologic stability of a previously qualified Master Reference.

1. The dating of a Master Reference may be extended beyond its expiration date by confirming its immunogenicity in a manner acceptable to APHIS.

2. A Master Reference can be requalified multiple times, provided that the stability of the Master Reference is shown to be maintained.

B. Requalification of a Master Reference should be done prior to the end of dating.

1. Products tested with expired Master References are not eligible for release.

2. Temporary extensions of dating may be granted by the CVB-LPD reviewer provided that the licensed manufacturer provides evidence of a protocol, plan, and continuing progress for accomplishing requalification. In general, such extensions shall not exceed 12 months and shall be supported by protocols and data acceptable and approved by APHIS. Such requests should be the exception and not routine practice.

C. Demonstrating the efficacy of a reference either directly or indirectly is one method of confirming immunologic stability.

D. Immunologic methods not requiring vaccination and challenge may demonstrate the stability of a reference, provided that the immunological response was initially correlated to protection during a host animal efficacy study.

E. An unfrozen product reference stored similarly to product can be directly requalified in animals.

F. Frozen product references, stabilized and frozen references, or purified references cannot be directly requalified in animals. The difference in composition and/or freeze-thaw cycle precludes direct requalification, as the continued correlation to product efficacy cannot be ensured.

G. For references that cannot be directly requalified in animals or where the manufacturer wishes to establish a Working Reference different from the Master Reference, a Qualifying Serial shall be used in requalification trials.

IX . QUALIFYING SERIAL

A. Qualifying Serials shall be produced in accordance with a filed Outline of Production.

B. Dilution of a production serial to produce a Qualifying Serial is allowed, provided that after dilution the relative concentration of all the components other than the agent being requalified is the same as per the filed Outline of Production.

C. Qualifying Serials should be produced within six (6) months prior to the immunogenicity trial, but a serial is acceptable if it was produced within the dating period of the product and as specified in the currently filed Outline of Production. The use of a recently produced Qualifying Serial ensures that the relationship of the reference to the product has not been altered by gradual changes in the production method or materials over time.

D. A serial prepared by a method which is different from the method specified in the currently filed Outline of Production is not acceptable for use as a Qualifying Serial.

E. Qualifying Serials used to requalify a Master Reference or Working Reference used in serial release testing shall have a relative potency less than or equal to 1.0 ([1.0) when compared to the Master Reference or Working Reference. A minimum of 5 independent replicate assays shall be used to confirm the relative potency value of the Qualifying Serial.

F. Qualifying Serials may be used as Working References, provided that:

1. The Qualifying Serial has a relative potency of 1.0 when compared to the Master Reference,

2. The Qualifying Serial is designated as the Working Reference in the filed Outline of Production or Special Outline.

G . The dating of Qualifying Serials used as Working References shall be:

1. Equal to the dating of the product, or

2. Equal to the dating allowed the Master Reference, if:

a. The relative potency of either the Qualifying Serial or Working Reference when compared with the Master Reference is equivalent to 1.0,

b. The equivalence of the Qualifying Serial/Master Reference relationship is monitored at intervals not less often than at mid-dating of the Master Reference using the *in vitro* assay specified in the filed Outline of Production or Special Outline, and

c. The monitoring plan is specified in the filed Outline of Production or Special Outline.

H. If the Qualifying Serial/Master Reference relationship is shown to be nonequivalent, the Qualifying Serial is no longer eligible for use in the test. In that event, the Master Reference may be designated as the Working Reference in the Outline of Production or Special Outline; or a new Working Reference may be qualified.

X. STORAGE OF A REFERENCE

A. The storage conditions for the Master or Working Reference used in an *in vitro* test for serial release should be stated in the filed Outline of Production or Special Outline.

B. Master References that are product references and stored in a manner similar to product should not require further dilution or bench-level manipulation prior to being used as Working References to test for serial release.

C. Master References that are purified antigens and stored in a manner similar to product may be diluted up to 1:100 prior to being used as Working References to test for serial release.

D. If the Master Reference is a product reference, stored frozen and later thawed for use as a Working Reference in an *in vitro* test for serial release, the following apply:

1. If the Master Reference, except for an aliquot which remains unfrozen, is frozen prior to initiation of the immunogenicity/protection study, no treatment of the serial is required if:

a. The unfrozen aliquot of the reference is used in the immunogenicity/protection study, and

b. Five (5) independent replicate assays of the *in vitro* test are used to establish the equivalency of the frozen and nonfrozen portions of the reference.

2. If the Master Reference is frozen after the initiation of the immunogenicity study, the serial being tested shall also be frozen and then thawed prior to the initiation of the *in vitro* test for serial release. For example, the samples selected for testing shall be frozen for a minimum of 24-48 hours prior to being thawed and tested for potency.

E. Master References that are purified antigens, frozen and later thawed for use as Working References in *in vitro* tests for serial release, can be used directly without the need for special treatment(s) if the reference was frozen prior to establishing the relative potency of the Qualifying Serial and the initiation of an animal immunogenicity study.

F. If the Master Reference is a purified antigen that is frozen after establishment of the relative potency of the Qualifying Serial used in the animal immunogenicity study, the equivalence of the relationships between 1) the frozen and never-frozen portions of the Master Reference, or 2) the frozen Master Reference and unfrozen Qualifying Serial must be demonstrated before the frozen Master Reference can be used as a Working Reference to test for serial release.

G. If stabilizers are used to extend the storage period of a frozen reference that is used as a Working Reference in an *in vitro* test for serial release, the following apply:

1. The stabilized frozen reference can be used in the *in vitro* potency test without further sample treatment if:

a. The stabilizer is added prior to freezing the reference,

b. The stabilizer is added to the reference prior to initiating the immunogenicity study,

c. A Qualifying Serial is used in the immunogenicity study, and

d. The relative potency of the stabilized and frozen Master Reference and the Qualifying Serial are equivalent when the immunogenicity study is initiated. A minimum of five (5) independent replicate assays in the *in vitro* test system shall be used to establish equivalence.

2. If stabilizer is added to the Master Reference after the initiation of the immunogenicity study, the *in vitro* test protocol should provide for similar treatment of the test sample. Treatment of the sample should be specified in the filed Outline of Production or Special Outline.

3. Mathematical corrections cannot be used to compensate for added stabilizer.

XI. REFERENCE REQUALIFICATION

The purpose of requalifying a Master Reference is to demonstrate its immunogenic stability and thereby extend the dating period for use for potency testing. Methods for demonstrating immunogenic stability include:

A. Host animal vaccination-challenge studies

1. Host animal protection studies in which a single dilution of the Master Reference or Qualifying Serial is administered by the least immunogenic of the recommended routes of vaccine administration may be used to extend the dating of the Master Reference for a period equal to that allowed for product dating.

2. Limiting dilution (dose-response) studies demonstrating an equivalence of protection at the beginning and end of the allowed dating of the reference are recommended in order to extend the dating of the reference for a period greater than that approved for product dating. When conducting limiting dilution studies, the following apply:

a. The antigen of interest should not be present in the diluent used for making dilutions of the Master Reference or Qualifying Serial.

b. Each dilution of the Master Reference or Qualifying Serial should be administered to a separate group of animals, and the dilutions that result in 50-percent protection in the separate studies compared. Based upon the two points a degradation curve is determined and used to estimate the stability of the Master Reference during the period of extended use.

c. Stability estimates based on two points require a very conservative interpretation of stability, but subsequent requalification studies should increase the accuracy of the estimate and provide a dating period closer to the extrapolated theoretical end of dating.

d. All evaluations should take into account statistical principles including sample size, variance, and confidence intervals.

B. Nonhost animal vaccination-challenge studies

1. Nonhost animal vaccination-challenge studies may be used to demonstrate the stability of the Master Reference if the protective immune response in nonhost animals is correlated to the protective response in host animals. Correlation may be established by:

a. Performing replicate dose-response studies in nonhost animals concurrently with a host animal protection study and again in nonhost animals at the end of dating in accordance with a protocol acceptable to APHIS. Stability would be determined by comparing the PD_{50} from the replicate dose response studies performed at the beginning and end of dating. Limiting dilution dose- response studies performed at the beginning and end of dating would be needed in order to demonstrate stability of the reference for greater than the allowable product dating, or

b. Citing references in the scientific peer-reviewed literature that recognize the nonhost animal model as acceptable to demonstrate host animal protection, provided that the model and supporting data are acceptable to APHIS.

C. Host animal serology studies

1. Serological response in the host animal may be used to requalify a Master Reference when a relationship between titer and protection has been established, as when:

a. A minimum protective titer for efficacy and/or potency has been published as part of the 9 CFR, Part 113, Standard Requirement for the product for which the Master Reference is being requalified,

b. Data from a protection study establish a relationship between titer and protection in a manner acceptable to APHIS, or

c. The scientific peer-reviewed literature recognizes a minimum serological titer as adequate to protect against an effective challenge, and the data are acceptable to APHIS.

2. A single study comparing the serological responses of animals used in the immunogenicity study to the responses of animals used in the requalification study may be adequate to demonstrate stability and extend the dating of a Master Reference for a period equal to that allowed for a serial of product.

3. Multiple comparative serological response studies demonstrating stability are needed to extend the dating of the Master Reference for periods longer than that allowed for product dating, with the following provisions:

a. The sera from previous qualification and/or requalification studies must be frozen for monitoring during subsequent titer determinations, or

b. Reference sera that have been characterized in a manner acceptable to APHIS are used to demonstrate the equivalence of test sensitivity and serological responses obtained at different times.

D. Nonhost animal serology studies

1. Serological response in nonhost animals may be used to demonstrate the immunologic stability of the Master Reference if:

a. Serological titers in nonhost animals are correlated to a protective response in host animals in replicate assays at the time of Master Reference qualification, or subsequently, in a manner acceptable to

APHIS, and

b. The immunologic response in nonhost animals is to a protective immunogen, and a dose-response relationship is demonstrated when dilutions of the Master Reference or Qualifying Serial are administered, or

c. The scientific peer-reviewed literature specifies a serological response in the nonhost animal as protective, and the supporting data are acceptable to APHIS.

2. A single requalification may be used to extend the dating of the Master Reference for a period equal to product dating.

3. Qualifying the Master Reference for longer dating requires demonstrating an equivalent serological response in the nonhost animal over time, with the following provisions:

a. The sera from previous Master Reference qualification/requalification studies must be frozen for monitoring during subsequent titer determinations, or

b. Reference sera that have been characterized in a manner acceptable to APHIS are used to demonstrate the equivalence over time of different test methods, test sensitivity, and serological responses.

E. Using Reference Standards to requalify the master reference

1. *In vitro* assays utilizing Master or Working References monitored by Reference Standards may be used if the criteria for utilizing the Reference Standard are specified in the filed Outline of Production or Special Outline, and

a. The Reference Standard contains the protective immunogen(s),

b. The Reference Standard is stored under quality control procedures acceptable to APHIS,

c. The stability of the Reference Standard is confirmed by animal studies not less frequently than every five (5) years in a manner acceptable to APHIS,

d. The relationship between the Reference Standard and the Master Reference is established, at the time of the host animal protection study, as parallel and linear by a minimum of five replicate assays using the *in vitro* assay specified in the filed Outline of Production or Special Outline, and is monitored at least yearly in a manner acceptable to APHIS, and

e. The range for the acceptable criteria is specified in a filed Outline of Production or Special Outline.

2. The Master Reference cannot be used as a Working Reference to release serials if monitoring, at any time, shows that the relationship between the Reference Standard and Master Reference is outside the specified limits. If found unacceptable, the Master Reference must be requalified or a new Master Reference prepared.

F. Using *in vitro* monitoring to requalify references

It is not permissible to requalify or extend the dating of a Master Reference using the following: *in vitro* data derived from monitoring internal references or non-reference standards; comparing optical densities; or comparing the same reference stored by different methods. However, such methods are recommended for detecting significant deterioration between requalifications. If deterioration is detected, immunological means should be used to determine if the reference may continue to be used for serial release testing. Monitoring procedures should be specified in filed Outlines of Production, Special Outlines, or protocols acceptable to APHIS.

XII. DATING OF A REFERENCE

A. The allowable dating of a Master Reference, whether purified antigen or product, stored in a manner similar to licensed product, is the same as the dating of a serial, except that the dating of the Master Reference may be extended if it is requalified in a manner acceptable to APHIS.

B. The expiration date of a Master Reference, purified antigen or product, that is stored frozen is the same as a serial of product, except that:

1. Five (5) years may be approved for frozen Master References that are monitored for stability, parallelism, and linearity in accordance with procedures that specify stability criteria in the filed Outline of Production or Special Outline, and

2. To confirm its immunologic stability, a Master Reference should be tested at mid-dating in a manner acceptable to APHIS. A Master Reference that is degrading at an unacceptable rate cannot be used as a Working Reference to release serials unless requalified using one of the appropriate methods specified in section XI.

3. There is no limit to the number of times a Master Reference may be requalified provided that it continues to demonstrate immunologic stability, parallelism and linearity, and appropriateness to the currently produced product as specified in the filed Outline of Production. It is not anticipated, however, that a Master Reference will be granted indefinite dating.

XIII. TESTING ADJUVANTED PRODUCT

A. When both the production serial and the Working Reference contain adjuvant, treatment to release bound adjuvant is allowed, provided that:

1. The test serial and the Working Reference receive similar treatment,

2. The treatment method is specified in the filed Outline of Production or Special Outline, and

3. The treatment method was demonstrated to be applicable at the time of an immunogenicity or Master Reference requalification study.

B. When adjuvant is present in the test serial but not in the Working Reference, treating the test serial to release bound antigen without having to similarly treat the Working Reference is allowed if:

1. The serial used in the immunogenicity test was treated, and

2. The treatment method is specified in the filed Outline of Production or Special Outline.

XIV. STATISTICAL METHODS

A. When using a parallel line assay the following minimum criteria have been established for relative potency assays that compare OD to the logarithm of the dilution:

1. First order linear regression lines are fitted to three or more contiguous dilutions. All OD values used in linear regression calculations must be m0.05 after subtracting the blank(s).

2. Lines determined from the first order linear regressions must have a correlation coefficient (r) of m0.95 to be considered valid.

3. The slopes of the lines should be significantly different (p [0.05) from zero using a one-sided Student's t-test.

4. The unknown line and the reference line must show parallelism (ratio of slopesm0.8 to [1.25) before a comparison can be made.

5. The best set of lines that fit the above criteria should be used to determine the relative potency of the unknown.

B. When there are three (3) or more replicates for each dilution, it may be possible to remove aberrant or outlier OD's. The mathematical criteria for removal must

be specified in the Outline of Production or Special Outline, and all outliers that meet the criteria must be removed. Operator discretion is not acceptable. If more than 40% of all OD values for a dilution are considered to be outliers, the dilution cannot be used in the calculation.

C. The relative potency of inactivated products may be calculated by comparing the antigen content of the test serial with a reference preparation using a parallel line immunoassay or equivalent method which measures linearity, specificity, and reproducibility in a manner acceptable to APHIS. There is available from the CVB-L an IBM or IBM-compatible computer program, The U.S. Department of Agriculture Veterinary Biologics Program's *Relative Potency Calculation Software*, that is based on these criteria. Supplemental Assay Method for Evaluation by the Relative Potency Method of *In Vitro* Enzyme Immunoassays Used in Testing of Veterinary Vaccines, SAM 318, details the method used in the program. This software and SAM, available free of charge, represent one implementation of a parallel line, relative potency evaluation method. When using a parallel line assay comparing the OD to other than the logarithm of the dilution, different criteria would be required for OD values transformed to different scales. Methods other than a parallel line assay will be considered, provided that linearity, specificity, and reproducibility criteria are at least as stringent as for the parallel line assay. Alternative methods should be submitted to the firm's CVB-LPD reviewer for consideration and approval. The method of determining the best set of lines and for calculating the relative potency shall be approved by APHIS and specified in the applicable Outline of Production or Special Outline.

/s/

Thomas E. Walton
Acting Deputy Administrator
Veterinary Services